

Selective Inhibition of Protein Targeting to the Apical Domain of MDCK Cells by Brefeldin A

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Abstract. Dipeptidyl peptidase IV (DPPIV) is mainly vectorially targeted to the apical surface in MDCK cells. BFA was found to abolish the apical targeting of DPPIV. This BFA effect could be achieved under conditions where the ER to Golgi transport and the total surface expression of DPPIV were essentially unaffected. BFA executed its effect during the transport from the *trans*-Golgi network (TGN) to the surface.

The inhibition of apical targeting resulted in enhanced mis-targeting to the basolateral surface. The mistargeted DPPIV was transcytosed back to the apical domain only after BFA withdrawal. In contrast, the basolateral targeting of uvomorulin was unaffected by BFA. These results established that the apical targeting of DPPIV was selectively abolished by BFA.

THE segregation of the plasma membrane in epithelial cells into the apical and the basolateral domains by tight junctions is essential for the vectorial functions of the epithelia. The apical and the basolateral domains are morphologically, functionally, and biochemically distinct. Both viral and cellular proteins that are confined to each domain have been identified and have been used to study the pathway and molecular mechanism for the biogenesis of domain-specific proteins (Simons and Fuller, 1985). It is generally believed that basolateral proteins are directly targeted to the basolateral domain from the *trans*-Golgi network (TGN).¹ The biogenesis of apical proteins is mediated by both direct transport from the TGN to the apical domain (direct or vectorial pathway) as well as by the transcytotic pathway that involves transient appearance on the basolateral domain followed by specific retrieval and transcytosis to the apical surface (Rodriguez-Boulant and Nelson, 1989; Simons and Fuller, 1985; Simons and Wandinger-Ness, 1990). Different apical proteins in the same cell type may have different pathways for their apical expression (LeBivic et al., 1990a; Matter et al., 1990). The same protein may take different routes in different cell types for the correct apical targeting (Bartles et al., 1987; Low et al., 1991a; Wessels et al., 1990). In MDCK cells, both endogenous and exogenous apical proteins are predominantly delivered to the apical surface by the vectorial pathway and the TGN plays a pivotal role in the sorting of apical and basolateral proteins (Caplan et al., 1986; Griffiths and Simons, 1986; Lisanti et al., 1989; Low et al., 1991b; Matlin

and Simons, 1984; Misek et al., 1984; Rindler et al., 1985; Rodriguez-Boulant et al., 1984). Furthermore, vesicles that mediate transport from the TGN to the apical or the basolateral surface have been isolated from MDCK cells and were shown to possess different protein compositions (Wandinger-Ness et al., 1990). Current studies are attempting to determine the nature of the signals that target proteins to the apical or the basolateral domain and to understand the molecular mechanism responsible for the correct targeting of domain-specific proteins.

BFA, a fungal metabolite, has recently been used extensively to study the exocytotic pathway (Pelham, 1991). It has been shown that BFA inhibits protein transport from the ER to the Golgi and caused disassembly of the Golgi apparatus with the redistribution of *cis*-, *medial*-, and *trans*-Golgi but not TGN markers back to the ER (Doms et al., 1989; Fujiwara et al., 1988; Lippincott-Schwartz et al., 1990; Magner and Paragiannes, 1988). Further experiments revealed that BFA caused the rapid dissociation of a 110-kD peripheral protein from the Golgi membrane (Donaldson et al., 1990). The Golgi to ER (retrograde) transport induced by BFA was most likely due to a breakdown of the Golgi apparatus and the fusion of the *cis*-, *medial*-, and *trans*-Golgi with the *cis*-Golgi network (CGN)¹. Fusion with the CGN resulted in their transport back to the ER by the pathway that normally recycles proteins from the CGN back to the ER (Lippincott-Schwartz et al., 1990, 1991). The 110-kD protein that was dissociated from the Golgi by BFA was later identified to be an essential component, named β -COP, of the non-clathrin-coated vesicles involved in anterograde vesicular transport (Duden et al., 1991). In vitro studies have shown that BFA could prevent the assembly of non-clathrin-coated buds in the Golgi cisternae. This could be the mechanism that mediates the inhibition of ER to Golgi transport observed in vivo. Furthermore, BFA also induced the forma-

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1. **Abbreviations used in this paper:** CGN, *cis*-Golgi network; DPPIV, dipeptidyl peptidase IV; endo H, endoglycosidase H; IgAR, IgA receptor; TGN, *trans*-Golgi network.

tion of extensive tubular networks that connected previously distinct Golgi cisternae into a single topological unit in the *in vitro* system. This observation could explain the breakdown of the Golgi apparatus and the fusion of the *cis*-, *medial*-, and *trans*-Golgi cisternae with the CGN followed by their redistribution back to the ER observed *in vivo* (Orci et al., 1991). Recent studies have also shown that BFA caused fusion of the TGN with the endosomal system (Lippincott-Schwartz et al., 1991; Woods et al., 1991).

We have previously observed that BFA could preferentially inhibit protein secretion from the apical domain of MDCK cells, resulting in enhanced secretion from the basolateral domain. This BFA effect could be achieved under conditions (0.5–2 $\mu\text{g/ml}$ of BFA) where the total protein secretion and the Golgi structure were essentially unaffected (Low et al., 1991c). This observation raised several intriguing questions. Whether the polarized targeting of membrane proteins was similarly affected? Where does BFA execute its effect in the cell? Could BFA be used to selectively and reversibly abolish the polarized protein trafficking in this epithelial cell? Could we gain more insights on the mechanism of polarized protein trafficking by employing BFA? The results described in this report provide some answers to these questions and demonstrate that BFA may provide a novel approach for further understanding of the molecular mechanism that mediates polarized targeting in this epithelial cell.

Materials and Methods

Materials

DME, FBS, and dialyzed FBS were purchased from Gibco Laboratories (Grand Island, NY). BFA was from Epicentre Technologies. [^{35}S]Met (>1,000 Ci/mmol) was from Amersham Corp. (Arlington Heights, IL). Goat anti-mouse IgG, streptavidin-agarose, and S-NHS-SS-biotin were obtained from Pierce Chemical Co. (Piscataway, NY). Transwells were from Costar (Cambridge, MA). Other reagents were from Sigma Chemical Co. (St. Louis, MO).

Cells and Cell Culture

Original MDCK (strain II) cells were kindly provided by Dr. Kai Simons (European Molecular Biology Laboratory, Heidelberg, Germany). MDCK cells expressing dipeptidyl peptidase IV (DPPIV) have previously been described (Low et al., 1991b). Cells were cultured in DMEM supplemented with 10% dialyzed FBS, 100 milliu/ml of penicillin, and 100 mg/ml of streptomycin. The medium was changed daily. Expression of the transgene was enhanced by culturing cells overnight in medium containing 10 mM sodium butyrate. The tightness of the cell monolayers grown on Transwells was checked as described (Low et al., 1991b).

Antibodies

mAbs against DPPIV were described before (Low et al., 1991b) and were generous gifts from Drs. D. L. Mendrick (Harvard Medical School, Cambridge, MA) and D. C. Hixson (Rhode Island Hospital).

Metabolic Labeling of Cells

After washing twice with PBS supplemented with 1 mM CaCl_2 and 1 mM MgCl_2 (PBSCM), cells were incubated for 45 min at 37°C in methionine-free medium containing 10% dialyzed FBS (labeling buffer). The cells were then pulse-labeled for 30 min with [^{35}S]Met (1 mCi/ml in labeling buffer). After washing (twice), the cells were then chased in medium containing excess cold methionine (100 mg/liter) for various times as detailed in each figure. For labeling cell monolayers grown on Transwell filters, 700 μl of labeling buffer containing [^{35}S]Met was added to the lower (basolateral) chamber, while the upper (apical) chamber received 700 μl of labeling buffer alone.

Selective Cell Surface Biotinylation

This was performed as described (Low et al., 1991b). Briefly, the tight cell monolayers were washed four times with PBSCM (5 min each) on ice. The side that was not being biotinylated received 1 ml of PBSCM. 1 ml PBSCM containing S-NHS-SS-biotin (0.5 mg/ml diluted from a 200 mg/ml stock in DMSO) was added to the side to be biotinylated. The biotinylation was performed twice on ice (20 min each) and stopped by repeated washing with PBSCM containing 50 mM NH_4Cl and/or culture medium with 10% FBS.

Surface Stripping by Reduction

This is performed as described using Cys (Low et al., 1991a). Briefly, 120 mg L-cysteine was added to a pre-mixed solution containing 9 ml water, 150 μl 5 M NaCl, 10 μl each 1 M CaCl_2 , and MgCl_2 in a 15-ml plastic tube. The solution was bubbled with nitrogen immediately, sealed, and stored on ice. 50 μl of 10 N NaOH and 1 ml FBS were added to the solution, mixed, and added to the cells immediately. Stripping was conducted twice on ice (25-min each).

Immunoprecipitation

This was performed exactly as described before (Low et al., 1991b).

Recovery of Biotinylated Proteins

The immunoprecipitate was boiled in 80 μl SDS sample buffer without β -mercaptoethanol, diluted immediately with 1 ml lysis buffer (25 mM Tris-HCl, pH 7.8, 250 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% BSA, 5% FBS, and 1 mM PMSF), and spun down in a microfuge. The supernatant was then incubated with streptavidin-agarose (100 μl 50% slurry) at 4°C for 90 min. After washing, the proteins were eluted by boiling for 5 min in 80- μl SDS sample buffer and analyzed by SDS-PAGE.

SDS-PAGE and Analysis of Fluorography

SDS-PAGE was performed as described (Low et al., 1991c). For [^{35}S]Met-labeled proteins, the gel was treated with 20% 2,5-diphenyloxazole in dimethyl sulfoxide for 2 h at room temperature before being dried for fluorography. This treatment enhanced the signals on the fluorograph significantly. The fluorograph was analyzed by scanning the gel with Visage 110 (BioImage, Eastman Kodak Co., Rochester, NY).

Results

Apical Domain Targeting of DPPIV Was Abolished by BFA

We have previously established MDCK cells (referred to as MDCK/D4 cells) that stably express DPPIV and vectorially target DPPIV to the apical plasma membrane (Low et al., 1991b). To examine the effect of BFA on polarized targeting of DPPIV, tight monolayers of MDCK/D4 cells were pulse labeled for 30 min with [^{35}S]Met and chased for 3 h in the absence or presence of different concentrations of BFA. The delivery of newly made DPPIV to the respective domains was then assessed (Fig. 1 A). Similar to previous observation, the majority of newly made DPPIV was delivered to the apical cell surface with a small portion being missorted to the basolateral domain (lanes 1 and 2) in the absence of BFA. This polarized targeting was unaffected by BFA at 0.1 $\mu\text{g/ml}$ (lanes 3 and 4). BFA at 0.5 $\mu\text{g/ml}$ partially affected this targeting (lanes 5 and 6). BFA at concentrations above 3 $\mu\text{g/ml}$ completely abolished the polarized targeting of DPPIV (lanes 7–12). Higher concentrations of BFA also reduced the total surface expression, especially at 10 and 30 $\mu\text{g/ml}$. We also observed that 1 $\mu\text{g/ml}$ of BFA could abolish the polarized targeting to a similar extent as 3 $\mu\text{g/ml}$ or higher concentrations of BFA (Fig. 1 D). To gain further insight into the effect of BFA on the polarized surface expres-

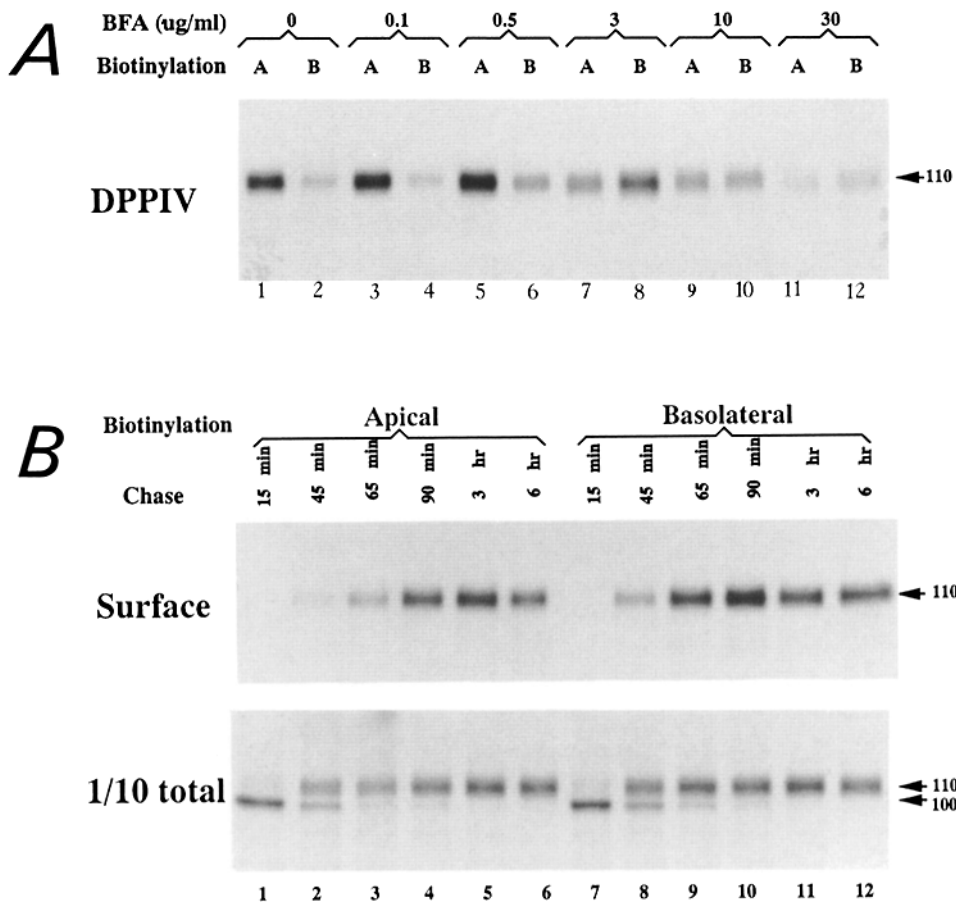
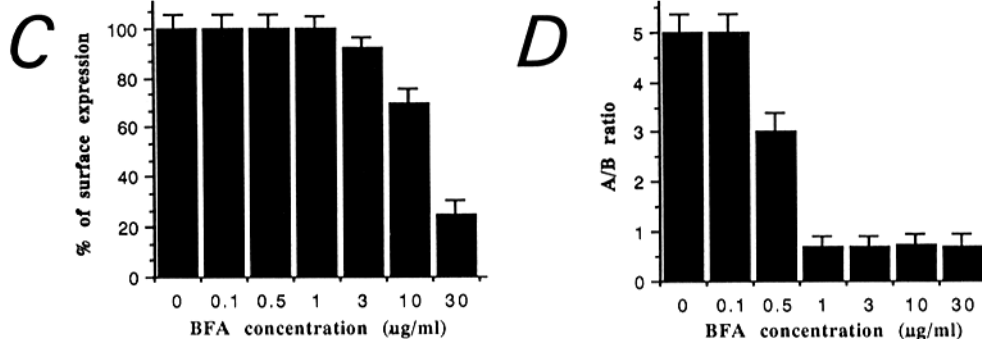


Figure 1. Effect of BFA on polarized surface expression of DPPIV. (A) Tight monolayers of MDCK/D4 cells grown on transwells were pulse labeled with [³⁵S]Met for 30 min followed by 3 h of chase in the absence or presence of BFA at the concentrations indicated. The apical (A) or the basolateral (B) surface was biotinylated with NHS-SS-biotin. DPPIV was immunoprecipitated from the cell lysates and the biotinylated DPPIV was recovered by absorption to streptavidin-agarose. After being resolved by SDS-PAGE, biotinylated [³⁵S]Met-labeled DPPIV was detected by fluorography. (B) Tight monolayers of MDCK/D4 cells were pulse labeled with [³⁵S]Met for 30 min followed by the chase periods indicated (1 µg/ml BFA was present during both pulse and chase periods). The apical (lanes 1-6) or the basolateral (lanes 7-12) surfaces were then biotinylated with NHS-SS-biotin. The cell lysates were immunoprecipitated. 1/10 of the immunoprecipitate was loaded onto the SDS-PAGE (1/10 total) and served as a measure of the total amount of newly made DPPIV. The remaining 9/10 of the immunoprecipitate was absorbed to streptavidin-agarose to recover biotinylated proteins and then analyzed (Surface), serving as a measure of the amount of protein transported to the respective plasma membrane domains. (C) Quantitation of BFA effect on total surface expression of DPPIV. The total surface DPPIV expression in the absence of BFA was arbitrarily defined as 100%, the total surface expression of DPPIV in the presence of various concentrations of BFA was normalized to that in the absence of BFA. (D) Quantitation of BFA effect on the polarity of DPPIV surface expression. The amount of DPPIV that was detected on the apical surface over that on the basolateral surface was defined as the A/B ratio and this was plotted as a function of BFA concentration.



arbitrarily defined as 100%, the total surface expression of DPPIV in the presence of various concentrations of BFA was normalized to that in the absence of BFA. (D) Quantitation of BFA effect on the polarity of DPPIV surface expression. The amount of DPPIV that was detected on the apical surface over that on the basolateral surface was defined as the A/B ratio and this was plotted as a function of BFA concentration.

sion, cells were pulse labeled for 30 min followed by various intervals of chase and the level of expression on the respective domain was examined. Using this approach, it was previously found that the majority of DPPIV was vectorially delivered to the apical domain (Low et al., 1991b). In the presence of 1 µg/ml of BFA, the polarized vectorial targeting of DPPIV was abolished and the amount of newly made DPPIV delivered to the basolateral domain was always slightly higher than that to the apical domain (Fig. 1 B). The results from Fig. 1, A and B (only 3-h chase point) were

quantitated and shown in Fig. 1, C and D. As seen, increasing concentrations of BFA beyond 3 µg/ml caused a gradual increase in inhibition of the total expression of DPPIV on the surface (Fig. 1 C), while the polarity of surface expression was completely abolished by BFA at 1 µg/ml or above (Fig. 1 D). The most important conclusion drawn from the above experiments is that BFA (at 1 µg/ml) could selectively disrupt the polarized targeting of DPPIV while the total surface expression was essentially unaffected. Previous studies using BFA at 0.5–2 µg/ml showed that the Golgi structure was ap-

parently unaffected (Low et al., 1991c). A recent study using both light and electron microscopy has also demonstrated that the Golgi apparatus was not affected by BFA around these concentrations (Hunziker et al., 1991b), although higher concentrations indeed caused a recycling of the Golgi back to the ER (Low et al., 1991c).

ER to Golgi Transport of DPPIV Was Unaffected by BFA at 1 $\mu\text{g/ml}$

Because previous studies using other cell types have shown that BFA selectively inhibits ER to Golgi transport (Doms et al., 1989; Fujiwara et al., 1988; Lippincott-Schwartz et al., 1990; Magner and Paragiannes, 1988), we examined the effect of BFA at 1 and 30 $\mu\text{g/ml}$ on the rate of ER to Golgi transport (Fig. 2). Newly made DPPIV in the ER has an apparent molecular mass of 100 kD and is sensitive to endoglycosidase H (endo H). This 100-kD form is efficiently

converted into a 110-kD form that is resistant to endo H due to modifications of its N-linked glycans by Golgi glycosylases and glycosyltransferases. In control cells (Fig. 2 A, no BFA), newly made DPPIV was completely transported to the Golgi structure within 60 min of chase. In cells treated with 1 $\mu\text{g/ml}$ of BFA, the rate of conversion of the 100-kD form into the 110-kD form was unaffected. In contrast, this conversion was significantly delayed with 30 $\mu\text{g/ml}$ of BFA. The quantitative result is presented in Fig. 2 B. These results demonstrate that the ER to Golgi transport of DPPIV was unaffected by 1 $\mu\text{g/ml}$ of BFA but significantly delayed by 30 $\mu\text{g/ml}$ of BFA. The disruption of polarized DPPIV targeting by 1 $\mu\text{g/ml}$ of BFA was, therefore, unlikely to be due to an effect on the ER to Golgi transport. Since 30 $\mu\text{g/ml}$ BFA could delay ER to Golgi transport, the observed inhibition on the total surface expression (in Fig. 1) by high concentrations (especially 10 and 30 $\mu\text{g/ml}$) of BFA was, at least in part, due to a delay in the ER to Golgi transport.

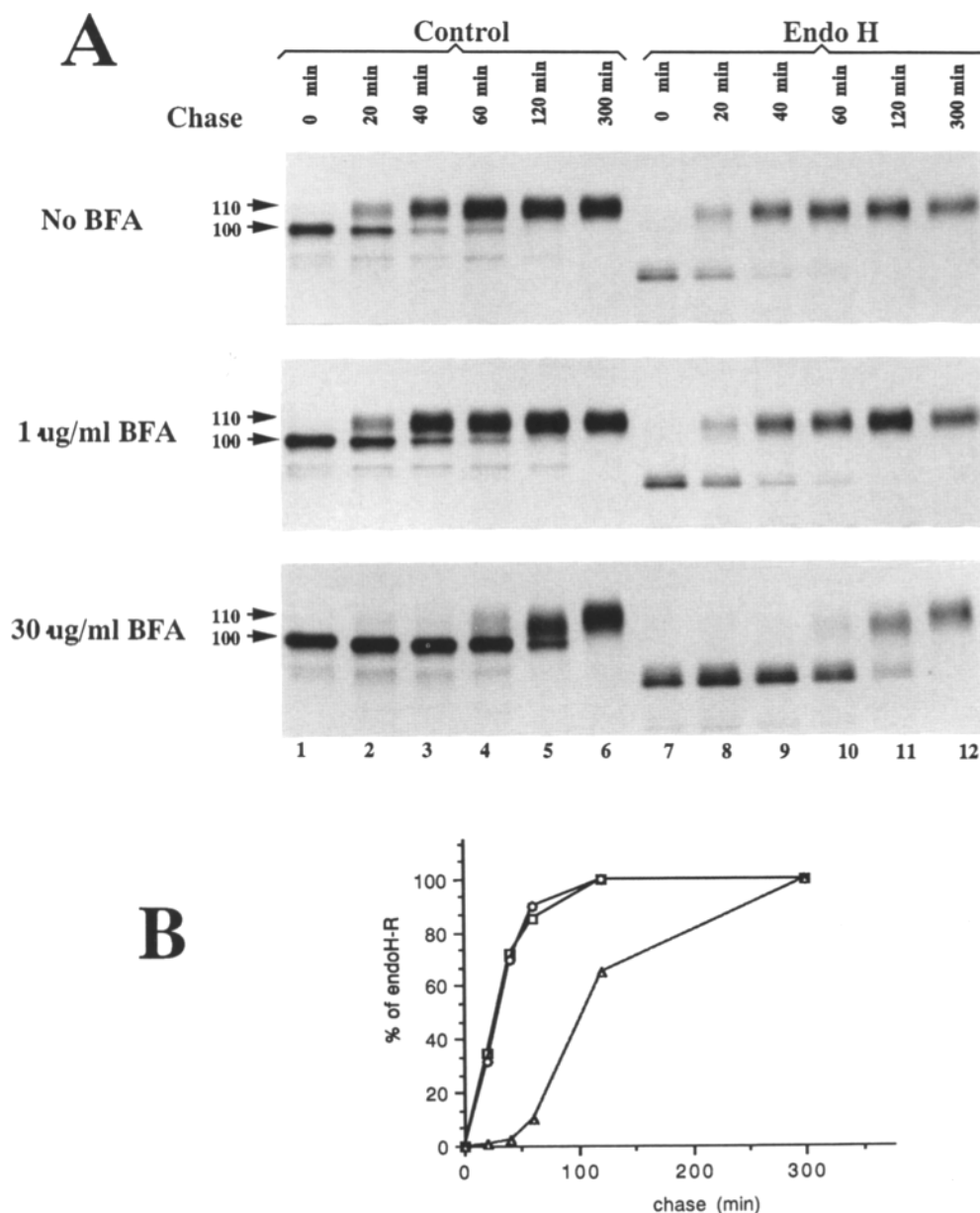


Figure 2. BFA effect on the transport of DPPIV from the ER to the Golgi apparatus. (A) MDCK/D4 cells were pulse labeled with [^{35}S]Met for 30 min followed by different chase periods as indicated. The pulse chase was performed either in the absence of BFA (*No BFA*) or in the presence of 1 or 30 $\mu\text{g/ml}$ BFA. DPPIV was then immunoprecipitated from the cell lysates. Half of the sample was treated with endo H while the other half was processed identically except that no endo H was added (*Control*). The 100-kD polypeptide is the endo H-sensitive ER form of DPPIV while the 110-kD polypeptide is the endo H-resistant Golgi form of DPPIV. (B) Quantitation of BFA effect on ER-Golgi transport. The percentage of endo H-resistant Golgi-form was plotted as a function of chase time. \circ , no BFA; \square , 1 $\mu\text{g/ml}$ BFA; \triangle , 30 $\mu\text{g/ml}$ BFA.

various concentrations of BFA at 20°C for an additional 60 min and then warmed up to 37°C for 60 min to allow transport from the TGN to the surface. The polarized surface expression was then examined (Fig. 3). Since TGN markers are not recycled back to the ER by BFA and the recycling of Golgi proteins in other cisternae caused by BFA are significantly blocked at 20°C (Lippincott-Schwartz et al., 1990), the newly made DPPIV, accumulated in the TGN under these conditions, would only be transported to the cell surface. As seen, the preferential apical targeting of DPPIV was similarly observed (lanes 1 and 2). BFA at 1 µg/ml or above abolished this polarized targeting (lanes 3–12). BFA at high concentrations (10 and especially 30 µg/ml) also re-

duced the total surface expression. These results demonstrate that BFA executes its effect on polarized targeting at the TGN and/or TGN to surface transport and that high concentrations of BFA also inhibit TGN to surface transport in addition to the disruption of polarized targeting.

BFA Effect Was Reversible

We next examined whether the BFA (1 µg/ml) effect on polarized targeting could be reversed by washing (Fig. 4). The details of the experiment are described in the legend and the results demonstrate that the BFA effect is indeed reversible. When the 3.5-h BFA treatment was shifted forward by 3 h

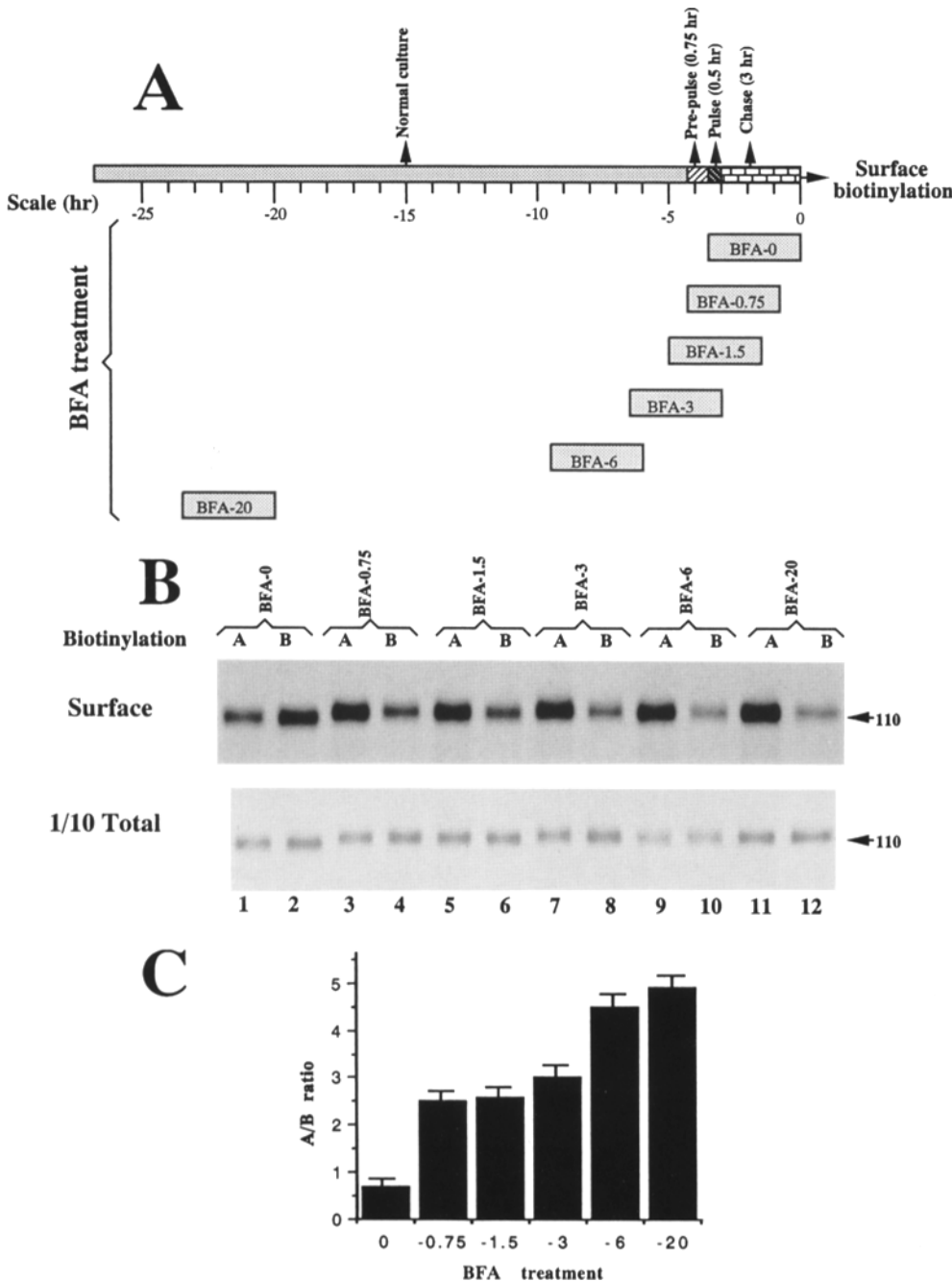


Figure 4. BFA effect on polarized DPPIV expression is reversible. (A) Polarized surface expression of DPPIV was assessed as described in Fig. 1, including a pre-pulse period of 45 min in which cells were incubated in medium without Met, a pulse period of 30 min in which cells were labeled with [³⁵S]Met, and a chase period of 3 h in which the cells were incubated in medium with excess unlabeled Met followed by selective surface biotinylation. For standard BFA treatment (as in Fig. 1 A), BFA was included in the pulse and chase period for a total of 3.5 h (BFA-0). For the reversal experiments, the 3.5 h BFA treatment was shifted forward by 0.75 h (BFA-0.75), 1.5 h (BFA-1.5), 3 h (BFA-3), 6 h (BFA-6), and 20 h (BFA-20) relative to the BFA-0. (B) Tight monolayers of MDCK/D4 cells were assessed for polarized surface expression of DPPIV under various conditions of BFA treatment. 1/10 Total represents 1/10 of the total immunoprecipitate resolved by SDS-PAGE and Surface represents the biotinylated surface DPPIV recovered from 9/10 of the immunoprecipitate by absorption to streptavidin-agarose. (C) Quantitation of data from B, showing the effect of different BFA treatments on the polarized surface expression of DPPIV. Note that the DPPIV in BFA-0.75, BFA-1.5, BFA-3, BFA-6, and BFA-20 has a slightly higher molecular mass as compared to BFA-0 with the most obvious size difference in BFA-3 onwards.

(BFA-3), in which the cells were treated for 3 h with BFA and then pulse labeled for 30 min in the presence of BFA followed by 3 h of chase without BFA, preferential apical targeting was clearly seen (Fig. 4 B, lanes 7 and 8). Almost complete recovery of polarized targeting was observed when the pulse-chase experiment was performed 2.5 h after the BFA treatment (BFA-6) (lanes 9 and 10). The quantitation of the data is presented in Fig. 4 C. Interestingly, the size of DPPIV was significantly increased in lanes 3–12 as compared to lanes 1 and 2 of BFA-0, with maximal size increase seen in BFA-3 onwards. Since the size of DPPIV in BFA-0 was identical to that in the control cells (Fig. 1), these observations suggest that BFA treatment followed by its withdrawal caused a significant increase in the size of newly made DPPIV.

BFA Treatment Withdrawal Caused Hyper-sialylation of Newly Made DPPIV

Since sialylation of N-linked glycosylation contributes significantly to the size of the glycan, we suspected that the increased size of DPPIV observed above was due to hyper-sialylation of the N-linked glycans. To test this, DPPIV was pulse labeled and chased under various conditions as outlined in Fig. 4 A. Immunoprecipitated DPPIV was either treated with neuraminidase or not treated. The samples were then analyzed by SDS-PAGE and fluorography (Fig. 5). As seen, the DPPIV produced in the presence of BFA (BFA-0) (lane 4) was of a similar size to that produced in the control

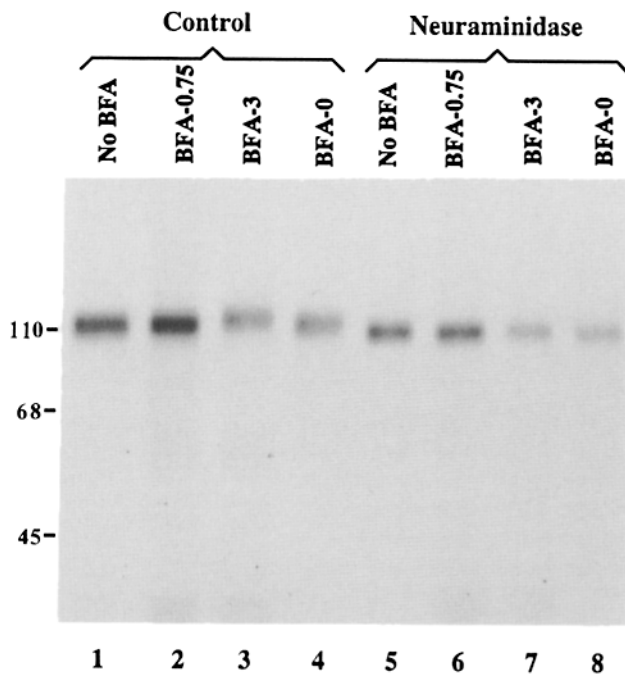


Figure 5. BFA treatment withdrawal caused hypersialylation of newly made DPPIV. MDCK/D4 cells were pulse labeled and chased under various conditions: *No BFA*, in the absence of BFA; *BFA-0.75*, *BFA-3* and *BFA-0* were as described in Fig. 4. [³⁵S]Methionine-labeled DPPIV was immunoprecipitated from the cell lysates. Half of the precipitate was treated with neuraminidase while the rest was processed identically except that no neuraminidase was included. As seen, neuraminidase converted the DPPIV produced under the different conditions into a polypeptide of identical size.

cells (*No BFA*, lane 1). For *BFA-0.75*, only a slight increase in size was detected (lane 2). *BFA-3*, however, resulted in a significant increase in size (lane 3), which is reproducible. When the samples were treated with neuraminidase, the DPPIV produced in all the conditions was converted into a polypeptide of the same size (lanes 5–8), demonstrating that the size difference was due to a difference in the extent of sialylation. Since the increase in size was only associated with DPPIV that was produced in cells that were pre-treated with BFA followed by its withdrawal, this demonstrates that BFA treatment withdrawal caused hyper-sialylation of the newly made DPPIV. The reason BFA treatment alone did not cause the hypersialylation and hypersialylation could only be observed after its withdrawal, is unknown.

Basolateral DPPIV Is Efficiently Transcytosed to the Apical Surface After BFA Withdrawal

As documented earlier, vectorial targeting of DPPIV was abolished by BFA (Figs. 1 and 3). One interesting question is whether the DPPIV, mistargeted to the basolateral surface (due to BFA treatment), could be transcytosed to the apical surface if BFA was removed. To examine this, cells were pulse labeled (30 min) and chased (3 h) in the presence of BFA so that ~55–60% of newly made DPPIV was mistargeted to the basolateral surface. The cells were then recultured at 37°C for various times and the amount of labeled DPPIV on the respective surface was assessed (Fig. 6 A). Without further culture at 37°C, the result was similar to Fig. 1 (lanes 1 and 2). Reculturing the cells for 1 h caused significantly higher amounts of DPPIV to be detected on the apical surface (lanes 3 and 4). Almost normal polarity was regained after 3–5 h of culture without BFA (lanes 5–8). In contrast, the polarity was not regained if the reculture (5 h) was performed in the presence of BFA (lanes 11 and 12). These results strongly suggest that the basolateral DPPIV was efficiently transcytosed back to its correct apical location after BFA withdrawal. This transcytosis was further confirmed by another experiment (Fig. 6 B). Cells were pulse labeled (30 min) and chased (3 h) in the presence of BFA and the basolaterally localized DPPIV was tagged with biotin. After warming up to 37°C for various times, the biotin was stripped off the DPPIV either from the apical or the basolateral surface, respectively. The amount that could be stripped off from the apical surface represented the amount that was transcytosed to the apical surface, while the amount that could not be stripped off from basolateral surface represented the amount that was endocytosed and/or transcytosed. As shown, a significant amount was transcytosed to the apical surface after 1 h of culture at 37°C (lane 5) and a majority of biotinylated basolateral DPPIV was accessible to stripping from the apical surface after reculturing for 3 h at 37°C (lane 8). The results for Fig. 6, A and B were quantitated in 6, C and D, respectively. These results demonstrate that mistargeted DPPIV could be correctly retargeted to the apical surface by transcytosis after removal of BFA. These results also demonstrate that BFA could inhibit the basolateral to apical transcytotic pathway of DPPIV.

Basolateral Targeting of Uvomorulin Was Unaffected by BFA

We next examined the effect of BFA on basolateral membrane protein targeting. Uvomorulin has been previously

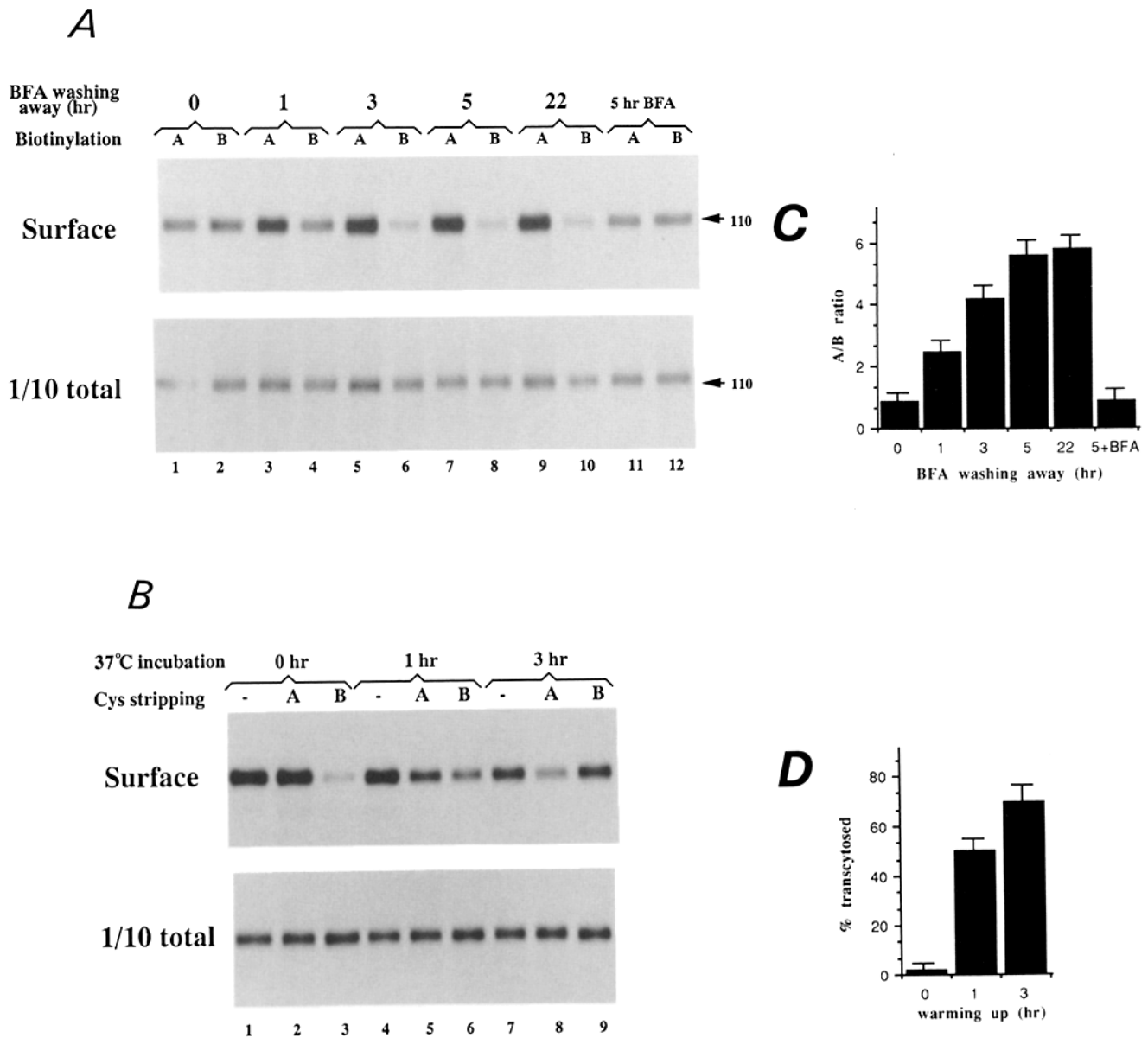


Figure 6. Transcytosis of DPPIV from the basolateral to the apical domain after BFA withdrawal. (A) Tight monolayers of cells were pulse-labeled with [³⁵S]Met for 30 min followed by 3 h of chase (both pulse and chase were performed in the presence of 1 μg/ml BFA). After washing away the BFA-containing media, cells were recultured at 37°C in the absence of BFA for various periods of time as indicated. Cells were then biotinylated on the respective surface and then processed for immunoprecipitation, absorption to streptavidin-agarose and SDS-PAGE. As a control, cells were also recultured in the presence of BFA for an additional 5 h (lanes 11 and 12). As shown, the correct surface polarity of DPPIV was resumed efficiently after BFA withdrawal (lanes 3–10) but not in the presence of BFA (lanes 11 and 12). (B) Tight monolayers of cells were pulse labeled with [³⁵S]Met for 30 min followed by 3 h of chase in the presence of 1 μg/ml of BFA. The basolateral surfaces were then biotinylated with NHS-SS-biotin. After reculturing the cells at 37°C for the times indicated, surface-associated biotin groups were either stripped off from the respective domains (A for apical and B for basolateral domain) by reducing with L-cysteine or not stripped (–). DPPIV was then immunoprecipitated from the cell lysates and biotinylated DPPIV was recovered by absorption to streptavidin-agarose from 9/10 of the immunoprecipitate. The amount that could not be stripped off from the basolateral surface served as a measure of the amount that was internalized and/or transcytosed, while the amount that could be stripped off from the apical surface served as a measure of the amount that was transcytosed from the basolateral to the apical surface. The results for A and B are quantified in C and D, respectively.

localized to the basolateral surface in MDCK cells and this basolateral localization was achieved by vectorial targeting (Le Bivic et al., 1990b). In MDCK/D4 cells, uvomorulin was similarly localized to the basolateral surface as assessed by immunofluorescence microscopy (data not shown). When

pulse-chase experiment was combined with selective surface biotinylation, it was found that uvomorulin was also vectorially targeted to the basolateral surface in MDCK/D4 cells (Fig. 7 A). Maximal expression on the basolateral surface was detected after 3 h of chase, and the surface uvomorulin

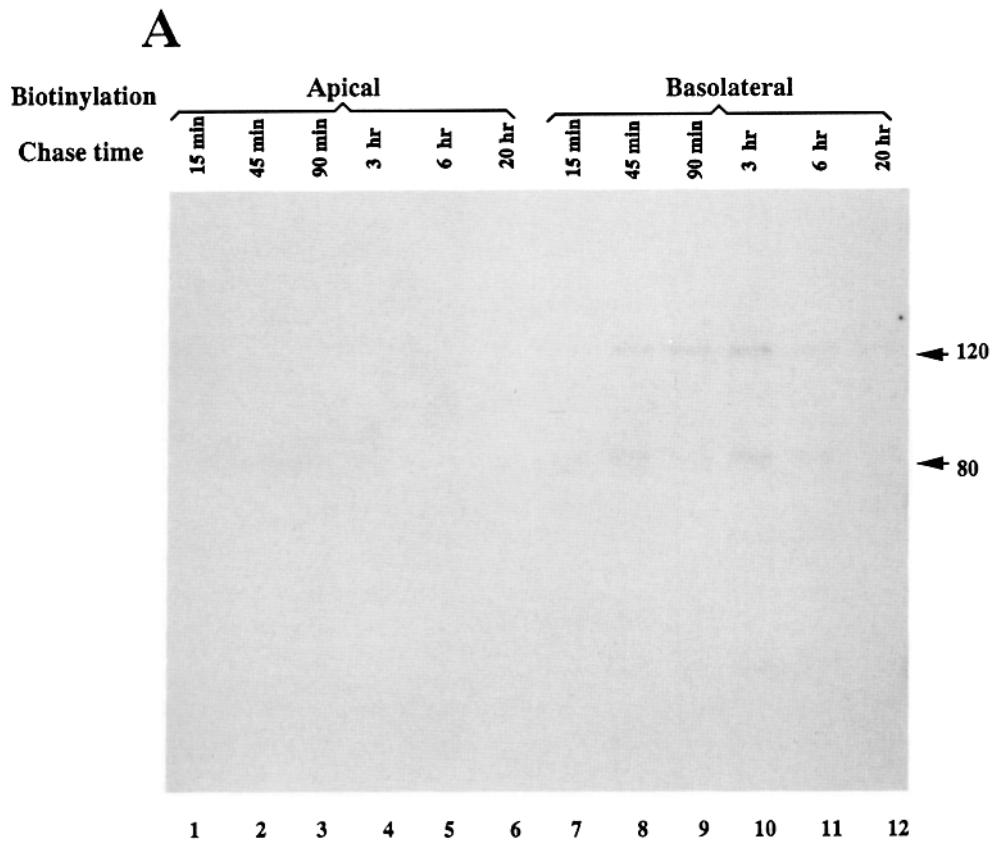
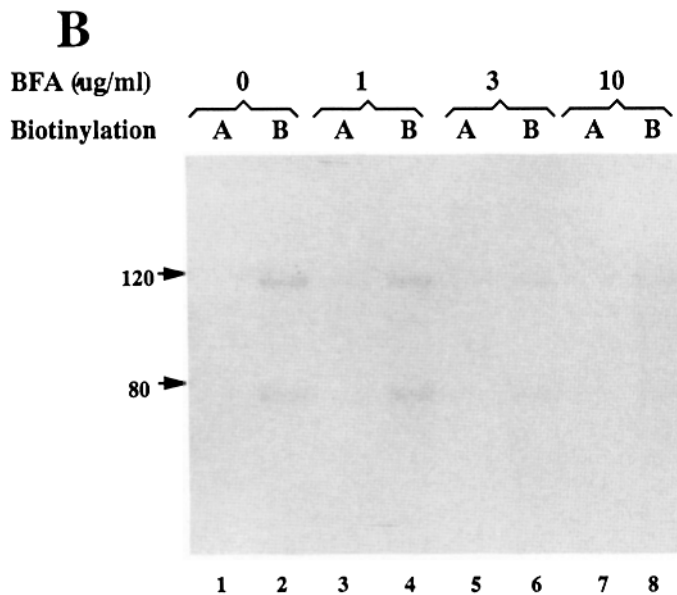


Figure 7. (A) Vectorial basolateral targeting of uvomorulin. Cell monolayers grown on filters were pulse labeled with [³⁵S]Met for 30 min and chased for various times followed by selective surface biotinylation as indicated. Biotinylated uvomorulin was recovered from immunoprecipitates and analyzed by SDS-PAGE and fluorography. (B) Basolateral targeting of uvomorulin was unaffected by BFA. Cells on filters were pulse labeled for 30 min and chased for 3 h. The pulse chase was performed either in the absence or presence of BFA at the indicated concentrations. The respective surface (A for apical and B for basolateral surface) was biotinylation. Cell lysates were immunoprecipitated with mAbs against uvomorulin. Immunoprecipitates were absorbed onto streptavidin-agarose and the absorbed biotinylated proteins were analyzed by SDS-PAGE. As seen, the basolateral targeting of uvomorulin was unaffected by BFA. The 120-kD polypeptide is the intact uvomorulin while the 80-kD polypeptide is most likely a proteolytic fragment of the intact uvomorulin.



turned over quite fast as revealed by the significant reduction of the surface uvomorulin after 6 h of chase. Uvomorulin was not detected on the apical surface. To examine the effect of BFA on uvomorulin targeting, cells were pulse labeled and chased for 3 h under various concentrations of BFA followed by selective surface biotinylation (Fig. 7 B). As can be seen,

the basolateral targeting of uvomorulin was unaffected by 1, 3, or 10 $\mu\text{g/ml}$ of BFA, although higher concentrations of BFA reduced the surface expression. These results demonstrate that BFA selectively abolished apical targeting of DPP-IV without apparent effect on the basolateral targeting of uvomorulin.

Discussion

Novel Effects of BFA

BFA was previously shown to inhibit ER to Golgi transport, to cause disassembly of the Golgi apparatus, and to recycle Golgi proteins back to the ER (Lippincott-Schwartz et al., 1990). Our previous experiments have demonstrated that BFA could selectively inhibit protein secretion from the apical surface (Low et al., 1991c). In the present study, we have extended this observation much further. It was revealed that the apical targeting of the membrane protein DPPIV was abolished by BFA and this effect could be achieved under conditions where total surface expression and ER to Golgi transport of DPPIV were unaffected. It was also found that BFA executed this effect at the TGN and/or TGN surface transport. The BFA effect on apical membrane protein targeting was selective because basolateral targeting of a membrane protein (uvomorulin) was unaffected under the same condition. Furthermore, BFA effect was reversible, which makes it suitable for the transient and selective perturbation of apical targeting in MDCK cells. Interestingly, hypersialylation of DPPIV was observed in cells that were pre-treated with BFA followed by its withdrawal. The hypersialylation was not seen in BFA-treated cells. Why withdrawal is required for BFA to cause hypersialylation is currently unknown and will be investigated in future experiments.

Transcytotic Targeting Pathway for Apical Proteins in MDCK Cells

Extensive studies have established that apical and basolateral membrane proteins (of both endogenous and heterogeneous origin) are sorted intracellularly and targeted vectorially to the respective domains in MDCK cells. One exception is the polymeric IgA receptor (IgAR), which is exocytotically targeted to the basolateral domain followed by transcytotic targeting to the apical domain (associated with the cleavage of the polypeptide from the membrane-spanning region) in liver hepatocytes as well as in transfected MDCK cells (Bomsel and Mostov, 1991). In hepatocytes as well as in cultured Caco-2 and LLC-PK1 cells, a transcytotic targeting pathway has also been revealed recently for apical proteins (Bartles et al., 1987; Low et al., 1991a, Le Bivic et al., 1990a; Matter et al., 1990). This raised the question as to whether a transcytotic targeting pathway for apical proteins was also present in MDCK cells. Since the IgAR is not a typical protein (Rodriguez-Boulant and Nelson, 1989; Bomsel and Mostov, 1991), the existence of a transcytotic targeting pathway for apical proteins in MDCK cells is currently unresolved. When expressed in MDCK cells, DPPIV was preferentially targeted to the apical domain with a small portion being mistargeted to the basolateral surface (Low et al., 1991b). Since the amount of missorted DPPIV was small, it was not possible to assess its final destination or to determine whether the missorted portion was eventually targeted to the apical surface by a transcytotic pathway (Low et al., 1991b). In the presence of BFA, ~55% of newly made DPPIV was transported to the basolateral surface. Since the BFA effect was reversible, withdrawal of BFA in these cells was used to assess the fate of the basolateral DPPIV. It was found that they were efficiently transcytosed back to the apical surface. These results suggest that there exists a transcytotic targeting

pathway for apical proteins in MDCK cells. Since the exocytotic targeting pathway for apical proteins is highly efficient in MDCK cells, the transcytotic targeting pathway contributes less to the apical targeting, unless the exocytotic targeting pathway becomes abolished by BFA (in the case of DPPIV) or is not operating due to a masking of the putative apical targeting signal (in the case of IgAR) (Bomsel and Mostov, 1991). Recently, it was shown that DPPIV missorted to the basolateral surface was efficiently transcytosed to the apical surface under normal conditions (Casanova et al., 1991b). These results, taken together, thus demonstrate that the sorting mechanism for apical proteins in MDCK cells exists in both the exocytotic and transcytotic pathways.

Transcytotic Apical Targeting Pathway Was Similarly Affected by BFA

The abolishment of the exocytotic apical targeting pathway caused enhanced mistargeting of DPPIV to the basolateral surface. The mistargeted DPPIV was transported back to the apical surface by the transcytotic targeting pathway after BFA was withdrawn. This did not occur in the presence of BFA. If BFA only inhibits the exocytotic targeting pathway, we should see a transient basolateral mistargeting of DPPIV followed by its transcytosis back to the apical surface. This was not observed. The prolonged existence of the mistargeted DPPIV on the basolateral surface in the presence of BFA and the mistargeted DPPIV being efficiently transcytosed back to the apical surface after BFA withdrawal, could only be explained by suggesting that the transcytotic targeting pathway was similarly affected by BFA. In support of this idea, a recent report has shown that the transcytotic pathway of the IgAR to the apical surface was selectively inhibited by BFA (Hunziker et al., 1991b). Furthermore, the inhibition by BFA of the transcytotic pathway for DPPIV, as well as that for the IgAR, indicates that the transcytosis of the IgAR is actually achieved by the normal transcytotic targeting pathway for apical proteins in MDCK cells.

Relatedness of Exocytotic and Transcytotic Targeting Pathways

The inhibition of both the exocytotic and transcytotic targeting pathways to the apical surface by BFA raises the question as to how these two pathways are related, in addition to their sharing the property of BFA sensitivity. This susceptibility to BFA suggests that these two pathways may use a similar or cross-related mechanism for the same apical targeting. Two possibilities exist. The sorting compartments for these two pathways are physically separated and related only functionally and biochemically. Alternatively, the sorting compartments for these two pathways may be physically integrated so that only one common sorting compartment exists, which integrates both the exocytotic and transcytotic targeting pathways. Newly made DPPIV was hypersialylated in cells that underwent BFA treatment withdrawal, while the basolateral DPPIV was not hypersialylated during transcytosis back to the apical surface under similar conditions of BFA treatment withdrawal. These results demonstrate that the transcytotic targeting pathway did not integrate with the Golgi compartment marked by the hypersialylation activity and imply that the sorting compartments for these two pathways are physically separated. However, the possibility that

they are physically integrated in a compartment distal to the compartment marked by sialyltransferases can not yet be excluded.

Default Pathway for Membrane Proteins May Exist for Both the Apical and Basolateral Surface in MDCK Cells

The BFA induced inhibition of the apical pathway for DPPIV caused its concomitant enhanced targeting to the basolateral surface. In contrast, targeting of a basolateral membrane protein (uvomorulin) was unaffected, suggesting that the basolateral pathway was intact in BFA treated cells. If basolateral transport was achieved only by a signal-mediated pathway, it would be difficult to explain the BFA-caused efficient transport of DPPIV to the basolateral surface. Alternatively, these observations could be explained by the existence of a basolateral default pathway for membrane proteins. The inhibition of apical targeting of DPPIV resulted in its enhanced incorporation into the basolateral default pathway. By contrast, a recent study reported that the cytoplasmic domain of several membrane proteins is required for their selective basolateral targeting and mutations in the cytoplasmic domain caused their mistargeting to the apical surface (Hunziker et al., 1991a). The structural motif for some basolateral proteins is enclosed in the region for efficient endocytosis via clathrin-coated pits (Hunziker et al., 1991a). Furthermore, introduction of an endocytotic signal into otherwise apical proteins resulted in their basolateral sorting (Brewer and Roth, 1991; Le Bivic et al., 1991). These results suggest that the endocytotic signal is or overlaps with the basolateral targeting signal in these proteins. Basolateral targeting signals that could be separated from the endocytotic signal have also been identified (Casanova et al., 1991a; Hunziker et al., 1991a). These results indicate the existence of a signal-mediated basolateral pathway and a default pathway for the apical surface. The basolateral signals identified could potentially function as a negative signal by excluding the proteins from other normal pathways. It should be noted that inhibition of apical targeting of DPPIV is never 100%, but with only about half of the protein being directed to each of the surfaces. This could be explained by the existence of a default pathway also for the apical surface, and inhibition of selective apical targeting of DPPIV by BFA caused it to be routed both to the apical as well as the basolateral default pathways with similar efficiency. Based on these observations, it could be proposed that default pathways may exist for both apical and basolateral surfaces in MDCK cells. The targeting to both apical and basolateral surfaces with comparable efficiencies for IgG Fc receptor isoform FcR2-B1 (which does not contain an endocytotic signal) and Igp120G-A (Hunziker et al., 1991a) is consistent with the presence of default pathways for both surfaces in MDCK cells. Further investigations will be required to gain more understanding about this issue.

Possible Mechanism of BFA Action

The inhibition of the apical but not basolateral targeting pathway suggests that the mechanism for the apical and basolateral targeting is distinct. Furthermore, this effect could be achieved under conditions where ER to Golgi transport and total surface expression were unaffected and the

Golgi apparatus was unaltered. Higher concentrations of BFA also caused an inhibition of ER to Golgi transport (Fig. 2) as well as the recycling of Golgi components back into the ER (Low et al., 1991c). These results demonstrate that the Golgi to apical surface transport is most sensitive to BFA while the ER-Golgi transport is less sensitive. Furthermore, we have demonstrated that BFA executes its effect on apical targeting at the TGN and/or TGN-apical surface transport. Previous studies suggest that inhibition of ER-Golgi transport by BFA may be mediated by dissociating β -COP from Golgi membranes and by inhibiting β -COP-mediated vesicle formation (Orci et al., 1991). β -COP belongs to a family of proteins, called adaptins, which are involved in the formation of various transport vesicles (Duden et al., 1991; Pearse and Robinson, 1990). One possibility is that distinct adaptins (with different BFA sensitivities) are involved in the formation of vesicles for ER-Golgi transport, TGN-apical surface transport, TGN-basolateral surface transport and transcytotic targeting. The adaptin, mediating TGN-apical surface transport and endocytotic apical targeting, was most sensitive to BFA, while adaptins mediating ER-Golgi and TGN-basolateral surface were less sensitive to BFA. This could be the explanation for the requirement of different concentrations of BFA for abolishing apical targeting and for inhibiting ER-Golgi transport. A BFA-resistant adaptin may mediate default pathways to both surfaces. Furthermore, the same or similar adaptin may be involved in the exocytotic and the endocytotic apical sorting pathways. Final proof for this hypothesis awaits the characterization of these adaptin molecules.

It was recently shown that BFA could induce the formation of cation channels in planar lipid bilayers (Zizi et al., 1991), whether this is related to in vivo effects has not yet been assessed. The high specificity of BFA and the different concentrations of BFA required for various effects in MDCK cells could not be simply explained by the formation of cation channels, although further investigations will be needed to reveal the mechanism of BFA effect in vivo.

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